Dairy Farm Reservoir of *Listeria monocytogenes* Sporadic and Epidemic Strains

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ABSTRACT

Identifying the reservoirs of a pathogen is vital for control of sporadic disease and epidemics. *Listeria monocytogenes* is a zoonotic foodborne pathogen that is responsible for 28% of food-related deaths in the United States annually, as well as a major cause of massive product recalls worldwide. To examine the role of the dairy farm as a potential source or reservoir for *L. monocytogenes* subtypes shown to cause human listeriosis, we compared the pulsed-field gel electrophoresis (PFGE) restriction enzyme digestion profiles of *L. monocytogenes* dairy farm–associated strains (milk, environmental, and bovine) to human sporadic and epidemic disease strains. Twenty-three percent of human sporadic strains had PFGE patterns identical to that of farm isolate(s). Additionally, three farm environmental strains and one human sporadic strain had a PFGE pattern identical to a strain of *L. monocytogenes* responsible for the 1985 California epidemic. These data indicate that this epidemic strain continues to cause sporadic human illness and has a potential dairy farm as a reservoir.

Listeria monocytogenes is a bacterial pathogen capable of causing significant morbidity and mortality in humans and animals. Human listeriosis accounts for approximately 2,500 clinical cases and 500 deaths each year in the United States *(14)*. Although clinical disease is relatively rare compared with other foodborne diseases, listeriosis may involve serious symptoms, including meningitis, septicemia, endocarditis, nonmeningitic central nervous system infection, febrile gastroenteritis, or abortion in people with predisposing conditions. Neonates, elderly people, and immunocompromised patients are particularly at risk.

Ready-to-eat meats, milk, and milk-related products have been implicated in several large outbreaks of listeriosis *(18)*. *L. monocytogenes* is widely distributed in the environment, and the source(s) of *L. monocytogenes* contamination of food products is not clearly understood. Raw milk has been suggested as one source of *L. monocytogenes* in the dairy processing environment *(7)*. However, because *L. monocytogenes* is killed by food treatment processes such as pasteurization, raw animal products are not believed to be a significant source of product contamination *(17)*. Nevertheless, persistent *L. monocytogenes* strains isolated repeatedly from bulk milk tanks *(15)* have been shown to form biofilms *(4)* and may serve as a source of persistent contaminants in the food processing environment, resulting in postprocessing contamination of food products.

In a recent investigation of three bovine listeriosis cases that occurred on two Pacific Northwest dairy farms, we discovered that the bovine clinical strains from both farms were closely related (one band difference when subtyped using pulsed-field gel electrophoresis [PFGE] and *Apa*I and *Asc*I digests). Additionally, the bovine clinical isolates were identical or closely related to silage and fecal strains present on the farms *(1)*. The farms were separated by a distance of approximately 50 miles, and no epidemiological link was identified. Therefore, we hypothesized that certain *L. monocytogenes* farm subtypes are endemic to the Pacific Northwest region and that these may serve as a reservoir for subtypes identified in human disease. To address this hypothesis, we compared the PFGE patterns of the strains obtained from the two dairy farms to the PFGE patterns of strains isolated from regional bulk milk tanks *(15)* and regional human sporadic listeriosis cases.

MATERIALS AND METHODS

Bacterial strains. Five hundred thirty-two *L. monocytogenes* isolates were obtained from 213 dairy farm samples collected during a 2-year period (Table 1). *L. monocytogenes* reference strains for PFGE subtyping were provided by Lewis Graves (Centers for Disease Control and Prevention, Atlanta, Ga.). *L. monocytogenes* strains associated with human sporadic cases were provided by the Washington State Department of Health, Shoreline, Wash., and human epidemics strains were obtained from International Life Sciences Institute (ILSI) *Listeria monocytogenes* strains collection via Dr. Martin Wiedmann (http://www.foodscience.cornell.edu/wiedmann/listeriadbase.htm).

***L. monocytogenes* environmental strain isolation and identification.** The method (MLG 8.03) detailed by the U.S. Department of Agriculture (USDA), Food Safety and Inspection Ser-

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TABLE 1. *Dairy farm environmental and clinical samples*

Sample type	Samples	Isolates	Strains[a]
Bovine clinical	2	2	2
Bovine fecal	90	320	43
Silage	52	90	20
Other[b]	69	120	23

[a] Strains were determined by PFGE subtyping (*Asc*I digest).

[b] Other farm samples include water, milk sock, bedding, feed, bird feces, and tractor blade swab.

vice (FSIS), Office of Public Health and Science (http://www.fsis.usda.gov/OPHS/microlab/mlgbook.htm) was used to isolate and identify *L. monocytogenes* strains from farm environmental samples. Samples were stored at 4°C before processing, and all samples were processed within 1 month of collection. A two-staged enrichment was used for strain isolation. Twenty-five grams or 25 ml of the sample was added to 225 ml of University of Vermont modified *Listeria* enrichment broth (Remel, Lenexa, Kans.), mixed well, and incubated at 30°C for 24 h. After mixing, 100 µl of the broth was transferred to 10 ml of Fraser broth with supplement (Difco, Becton Dickinson, Sparks, Md.) and incubated for a maximum of 48 h at 30°C. At the 24-h point and then again at the 48-h point, 50 µl from positive Fraser broth tubes were plated on modified Oxford agar base with supplement (Remel) and incubated for 24 to 48 h at 30°C. Colonies phenotypically characteristic of *L. monocytogenes* were spotted onto ALOA plates (Microbiology International, Frederick, Md.) and incubated at 30 to 37°C for 48 h to test for phospholipase activity. Phospholipase-positive isolates were then CAMP tested with *Staphylococcus aureus* using tryptic soy agar plates with 5% sheep blood (USDA, FSIS, MLG8.6.3) and tested for motility in brain heart infusion with 0.4% agar (30°C for 24 h). PCR was used to verify the presence of the listeriolysin gene *(3)* for any isolate that was positive for all of the tests.

Serotyping. Denka Seiken *Listeria* antisera (Tokyo, Japan) were obtained from Accurate Scientific (Westbury, N.Y.). Serotyping was performed according to the manufacturer's recommendations.

PFGE. Strain typing using the 30-h PFGE protocol was performed as previously described *(8)*. Four to eight isolates from each positive sample were subtyped. Two reference strains (H2446 and F2365) were used to ensure uniform DNA preparation among experiments. Lambda size standard (Bio-Rad, Hercules, Calif.)

was used as a molecular weight marker. BioNumerics (Applied Maths, Kortrijk, Belgium) was used for band detection and construction of dendrograms using Dice binary coefficients and unweighted pair group method using arithmetic averages (UPGMA). Visual inspection of band identification was performed following BioNumerics' band assignment. A 1.5% tolerance was applied to band matching. Reference strains (H2446 and F2365) were included in each gel to minimize gel-to-gel variability. Gels in which the standard isolates did not cluster within 95% similarity in the dendrogram were rejected from the analysis. Restriction enzyme digestion profiles (REDPs) that had more than 95% similarity according to BioNumerics were visually inspected to ensure similarity. Isolates that had identical REDPs using *Asc*I were analyzed using a second enzyme (*Apa*I).

RESULTS

A recent investigation of bovine listeriosis cases that occurred on two Pacific Northwest dairy farms demonstrated that the bovine clinical isolates from both farms were closely related and that these strains were identical or closely related to silage and fecal strains present on the farms (Fig. 1). To examine the role of the dairy farm as a potential source or reservoir for *L. monocytogenes* subtypes shown to cause human listeriosis, we compared the PFGE restriction enzyme digestion profiles of *L. monocytogenes* strains obtained from U.S. Pacific Northwest bulk milk ($n = 29$) *(15)*, dairy farm environmental ($n = 58$), and bovine listeriosis samples ($n = 2$) to human sporadic disease strains ($n = 30$) obtained from the Washington State Department of Health. Nine bulk milk strains (31.0%) had PFGE REDP patterns (*Apa*I and *Asc*I) identical to those obtained from the two dairy farms, indicating that clones present on the two dairy farms also had a more widespread regional distribution.

On comparing the farm-associated strains (milk, environmental, and bovine) to regional human sporadic listeriosis strains, we found that seven human sporadic strains (23.3%) had PFGE patterns identical to those of farm isolate(s). These data suggest that the dairy farm is a potential source of contamination in food processing plants, ultimately serving as a reservoir for product contamination and human listeriosis.

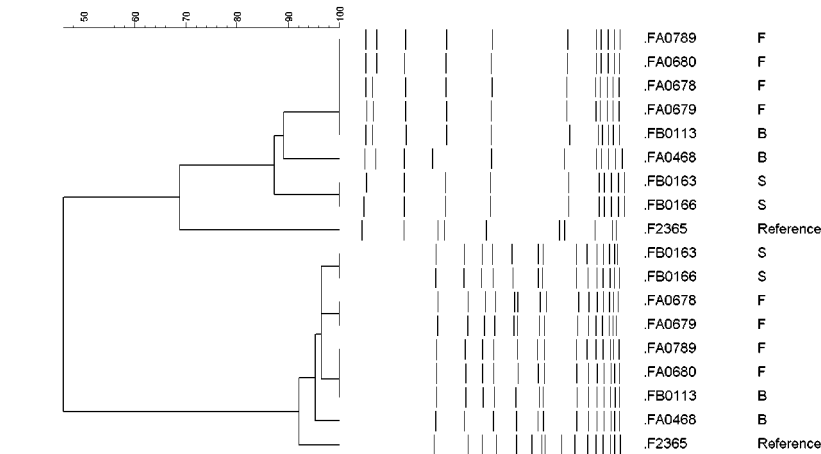One of the control strains we include as a standard reference in every PFGE analysis is a strain from the 1985

FIGURE 1. *Comparison of* Asc*I and* Apa*I REDPs from* L. monocytogenes *bovine clinical (B), fecal (F), and silage (S) strains obtained from farm A (FA) and farm B (FB). The upper cluster consists of the* Asc*I REDP and the lower cluster consists of the* Apa*I REDP. The UPGMA dendrogram was constructed from Dice coefficients with 1.5% band matching tolerance.*
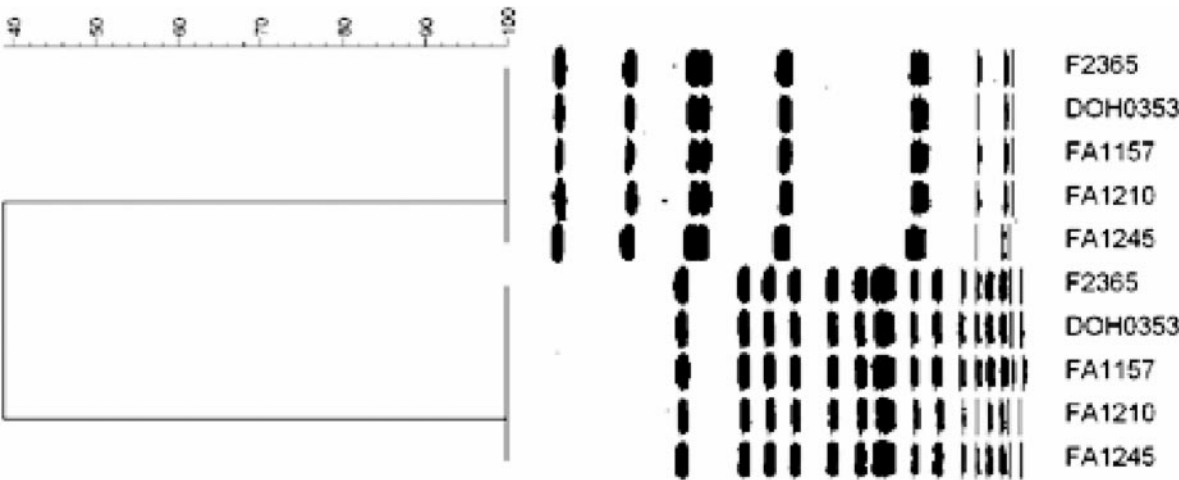
FIGURE 2. *Comparison of farm, human sporadic, and epidemic strain F2365 PFGE AscI and ApaI patterns. The upper cluster consists of the AscI REDP and the lower cluster consists of the ApaI REDP. FA1157, FA1210, and FA1245 are strains isolated from silage (FA1157, FA1210) and feed (FA1245). DOH0353 is a human sporadic strain obtained from Washington State Department of Health.*

DISCUSSION

Jalisco cheese outbreak in California (strain F2365). Interestingly, we found that this strain was identical to three farm environmental strains and one human sporadic strain (Fig. 2, Table 2). These data indicate that this epidemic-associated subtype continues to cause sporadic human illness and suggests the dairy farm as a reservoir.

Other epidemic strains were subsequently compared with regional farm and human sporadic strains using PFGE subtyping (Table 2). The serotype 4b epidemic strain from the 1983 Massachusetts outbreak (ILSI 1) was identical to a food isolate (ILSI 29) from the United Kingdom 1988 to 1990 epidemic and a regional sporadic human isolate. The serotype 1/2a epidemic strain from the U.S. multistate 2000 outbreak associated with sliced turkey deli meat (ILSI 35) was identical to a human sporadic strain (ILSI 34) and the associated hot dog isolate (ILSI 33) and a regional sporadic human strain. The serotype 4b strain from the North Carolina outbreak in 2000 (ILSI 37, 38) did not have an identical match with any regional strains nor did the epidemic strain from the 1998 to 1999 multistate hot dog–associated outbreak (ILSI 2). These data suggest that epidemic strains other than strain F2365 may also be geographically widespread but may not have an on-farm reservoir.

TABLE 2. *Comparison of epidemic strains to sporadic and farm-associated subtypes*

PFGE subtype[a]	Epidemic	Sporadic	Farm associated
A	F2365	DOH0353	FA1157
			FA1210
			FA1245
B	ILSI 1	DOH1264	NM[b]
	ILSI 29		
C	ILSI 35	ILSI 34	NM
D	ILSI 37/38	NM	NM
E	ILSI 2	NM	NM

[a] Strain subtypes were designated using *AscI* and *ApaI* digests.
[b] NM, no matching strain identified.

Results of this study support the hypothesis that certain *L. monocytogenes* farm subtypes are endemic to the Pacific Northwest region and that these strains serve as a reservoir for subtypes identified in human disease. Because no epidemiological link between farms (shared source of silage or water, common veterinarian) was identified, how strains are spread among farms is unclear. Common feed sources, movement of animals and/or personnel among farms, and spread of strains by birds or other wildlife are possible explanations for this observation.

Although listeriosis is a zoonotic disease, the primary reservoir of *L. monocytogenes* in food processing plants is believed to be environmental strains that colonize the plants *(7)*. Therefore, the relatively high percentage of human sporadic strains that shared a PFGE subtype with farm subtypes was surprising. These data suggest that raw materials contribute to environmental strains that colonize food processing plants and serve as a source for postprocessing contamination of food.

Many strains that cause large epidemics have been shown by genetic subtyping to be closely related. Specifically, four epidemic clonal groups have been identified and are referred to as epidemic clones I, Ia, II, and III (ECI, ECIa, ECII, and ECIII) *(1, 13, 16)*. Strain F2365 is a member of ECI, and this clonal group is widespread geographically and has caused outbreaks in both North America and Europe. Boerlin and Piffarretti *(2)* used multilocus enzyme electrophoresis (MLEE) to show that this clonal group is frequently associated with animals and humans in Switzerland and hypothesized that animals may serve a dissemination mechanism. However, ECI strains are less commonly isolated from food *(2, 6, 13)*. The results of the present study agree with those of Boerlin and Piffarretti *(2)*, and importantly PFGE subtyping has been shown to have higher resolution compared with MLEE *(5, 9)*.

Although strain F2365 was previously associated with a large human epidemic, the clones present on the farm

were not the cause of the bovine listeriosis cases analyzed nor were they associated with a recent regional human epidemic. Possible explanations for this include factors such as inadequate dose, differences in host immune status, strain preadaptation *(17),* or, in the case of bovine listeriosis, route of inoculation *(20).* In addition, seemingly unrelated sporadic cases may have an unrecognized common source. Alternatively, there may be genetic differences present in the clones that are not identified by PFGE analysis.

Although it has been previously documented that human and animal isolates may share the same subtype *(10– 12, 19),* to our knowledge, this is the first study that describes the presence of human epidemic strain F2365 on the farm. The results of this research indicate that because dairy farm–associated contaminants serve as a reservoir for human disease, food processors should be vigilant in controlling the spread of raw materials in the processing plant. These results also suggest that on-farm management strategies that reduce or eliminate the occurrence of farm reservoirs of *L. monocytogenes* are important to the reduction of risk for listeriosis in humans.

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